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Hung Lee

University of Guelph

Audrey L. Atkin

University of Nebraska-Lincoln, aatkin@unl.edu

Maria F.S. Barbosa

National Research Council of Canada

Delbert R. Dorscheid

National Research Council of Canada

Henry Schneider

National Research Council of Canada

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Effect of biotin limitation on the conversion of xylose to ethanol and xylitol by *Pachysolen tannophilus* and *Candida guilliermondii*

Hung Lee,¹ Audrey L. Atkin,² Maria F. S. Barbosa,^{2*} Delbert R. Dorscheid,² and Henry Schneider²

1. Department of Environmental Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

2. National Research Council of Canada, Division of Biological Sciences, Ottawa, Ontario K1A 0R6, Canada

* Visiting scientist from the Biotechnology Division, Foundation for Industrial Technology, Sao Paulo, Brazil.

Corresponding author – Henry Schneider, National Research Council of Canada, Division of Biological Sciences, Ottawa, Ontario K1A 0R6, Canada

Abstract

The relative amount of ethanol and xylitol accumulated in aerobic batch cultures of *Pachysolen tannophilus* and *Candida guilliermondii* on D-xylose depended on the extent of limitation by biotin. In high biotin media *P. tannophilus* favored ethanol production over that of xylitol while *C. guilliermondii* favored xylitol formation. However, as the extent of biotin limitation increased, the ratio of ethanol to xylitol produced by both organisms increased. The results are of interest in efforts to control such ratios.

Keywords: growth, limitation, biotin, D-xylose, ethanol, xylitol, yeast, *Pachysolen tannophilus*, *Candida guilliermondii*

Introduction

Ethanol and xylitol are potentially useful compounds that a number of yeasts can produce from D-xylose. Some of these organisms can produce relatively high yields of ethanol under certain conditions, but xylitol is often coproduced.^{1,2} Others can produce largely xylitol, but some ethanol can also be present.³ Process development for the production of either of these compounds would be simplified if only one were produced preferentially or exclusively.

The formation of ethanol and xylitol from D-xylose by several yeasts has been suggested to be associated with the limitation of growth, and hence, to act as secondary metabolites.⁴ The limiting factor(s) responsible for the onset of accumulation of these compounds in the medium is unclear, although oxygen can play a role.⁵⁻⁸ Moreover, while a number of factors can influence the relative amounts of ethanol and xylitol formed, the mechanisms involved are understood imperfectly. The rate of oxygen use⁵⁻⁸ and nitrogen source⁹ can alter ethanol yield as well as the ratio of ethanol to xylitol. Addition of azide¹⁰ or polyethylene glycol¹¹ can increase ethanol concentration while decreasing that of xylitol.

The present study investigated effects on ethanol and xylitol accumulation caused by limiting growth to various extents by biotin, a vitamin for which many yeasts are auxotrophic. The objective was to investigate effects on product ratio of the limitation of a nutrient other than oxygen that was relatively inexpensive and was required in only small amounts. A stimulatory effect of biotin on ethanol formation from D-xylose was found in *Candida shehatae*.¹² Biotin effects were investigated in two species that had differing tendencies to form the products of interest; *Pachysolen tannophilus*, which can produce relatively high yields of ethanol under some conditions^{13,14} and *C. guilliermondii*, which can produce relatively high concentrations of xylitol. Large differences were found with the two yeasts in both the absolute and relative amounts of ethanol and xylitol accumulated in batch cultures as the extent of biotin limitation varied, but both tended in the direction of producing relatively more ethanol as biotin concentration decreased.

Materials and methods

Microorganisms

P. tannophilus NRRL Y 2460 and *C. guilliermondii* FTI-20037 were maintained at 4°C on plates consisting of 2% agar, 2% yeast extract, 1% peptone, and 2% glucose (YEPD). The latter strain was obtained from the Foundation for Industrial Technology, Sao Paulo, Brazil.

Biotin limited media

These contained 4.0 or 4.3% (w/v) D-xylose as specified, 0.43% (w/v) ammonium sulfate, and the salts, vitamins, and trace elements in yeast nitrogen base (Difco), with the follow-

ing exceptions. Biotin was present in amounts ranging from zero to $2 \mu\text{g l}^{-1}$, and magnesium chloride was substituted for magnesium sulfate. The stock solutions of *p*-amino benzoic acid, riboflavin, and thiamine HCL used to prepare the media were kept cold and shielded from light. Sterilization, as in all other instances, was accomplished by filtration.

Inocula

A loopful of cells from a YEPD plate was placed in 100 ml of 0.67% yeast nitrogen base without amino acids plus 2% D-xylose kept in loosely capped 250 ml flasks on a gyratory shaker at 200 cycles per min and 30°C . The cells were harvested after 48 h by centrifugation at 8000g and washed twice with distilled water. The final cell pellet was then resuspended in 100 ml of distilled water.

Growth limitation experiments

A volume of 0.1 ml of the inoculum was placed into 100 ml of the biotin test media, which were kept in loosely capped 250 ml flasks and shaken as described above. At intervals, samples were removed to monitor growth and for product analyses.

Analytical methods

Growth was followed at 600 nm using 1 cm cuvettes and a Beckman model 35 Spectrophotometer. Where necessary, samples were diluted before measurement, a procedure that for *P. tannophilus* provides a linear relationship between dry weight and absorbance.¹⁵ Before product analysis, cells were removed by centrifugation in an Eppendorf model 5412 centrifuge. Ethanol was determined by gas liquid chromatography (g.l.c.)¹⁶ and sugars by high performance liquid chromatography (h.p.l.c.) using a BioRad HPX 87P column with water as eluant.

Results

Biotin limitation decreased the extent of growth and rate of xylose utilization of both yeasts (fig. 1 and 2). These decreases generally became larger as the initial concentration of biotin decreased. *P. tannophilus* had a greater requirement for biotin than *C. guilliermondii*, as indicated by the more extensive use of xylose by the latter at two of the lower concentrations of biotin employed, 0.05 and $0.1 \mu\text{g l}^{-1}$. Growth of *P. tannophilus* approached the maximum for the medium used with $2.0 \mu\text{g l}^{-1}$ biotin while the corresponding limit with *C. guilliermondii* was $1.0 \mu\text{g l}^{-1}$.

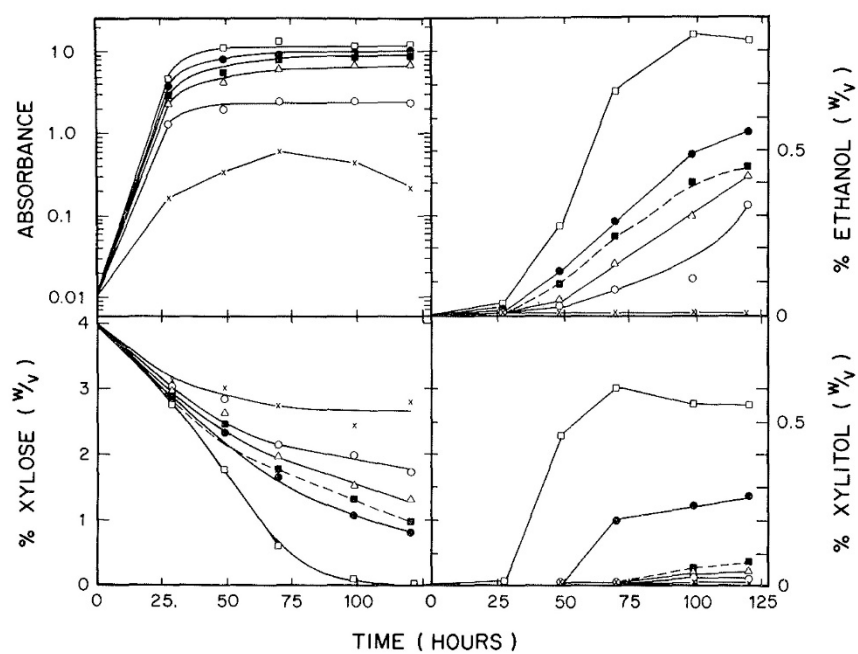


Figure 1. Effect of initial biotin concentration on *P. tannophilus* cultures. Concentrations in $\mu\text{g l}^{-1}$: x, 0; o, 0.05; Δ , 0.10; \blacksquare , 0.15; \bullet , 0.20; \square , 2.0.

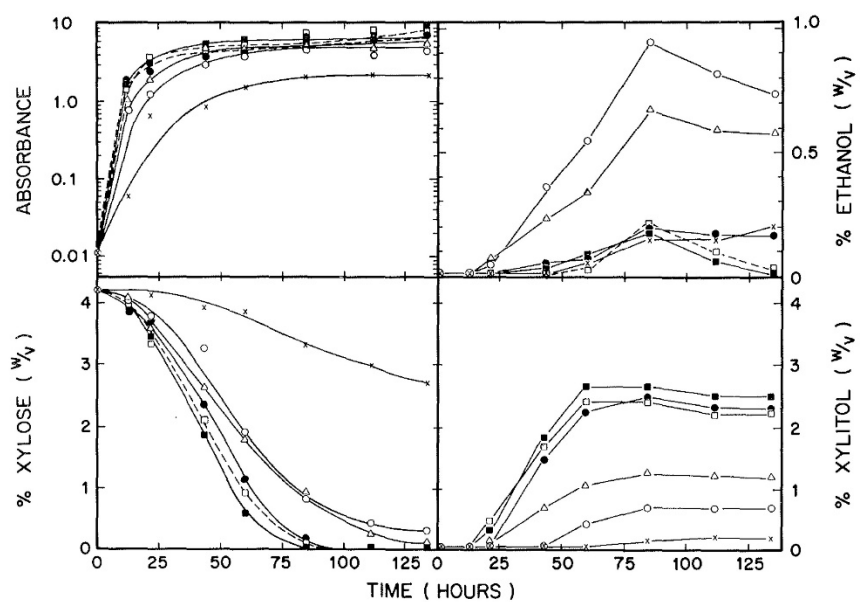


Figure 2. Effect of initial biotin concentration on *C. guilliermondii* cultures. Concentrations in $\mu\text{g l}^{-1}$: x, 0; o, 0.05; Δ , 0.10; \blacksquare , 0.25; \bullet , 0.50; \square , 1.0.

The two yeasts differed appreciably in the relative amounts of xylitol and ethanol produced at the highest concentrations of biotin in the medium employed. *C. guilliermondii* produced xylitol preferentially, maximum values of 2.4% xylitol and 0.2% ethanol, while *P. tannophilus* produced ethanol preferentially, peak values of 0.6% for xylitol and 0.185% for ethanol (fig. 1 and 2).

The concentration of ethanol and xylitol accumulated in cultures of both yeasts was altered as the initial concentration of biotin decreased (fig. 1 and 2). However, the point of prime interest in the present study was that, with both organisms, as biotin concentration decreased, the relative concentrations of ethanol and xylitol produced changed in the direction favoring ethanol (fig. 3 and 4). The changes in product ratio resulted from differences in the dependence on the initial biotin concentrations of the concentrations of ethanol and xylitol accumulated. With *P. tannophilus*, the concentration of xylitol found in the medium increased abruptly at about $0.15 \mu\text{g l}^{-1}$ biotin, while the corresponding increase in ethanol concentration was linear (fig. 3). With *C. guilliermondii*, xylitol began to decrease appreciably below $0.25 \mu\text{g l}^{-1}$ biotin while ethanol increased (fig. 4).

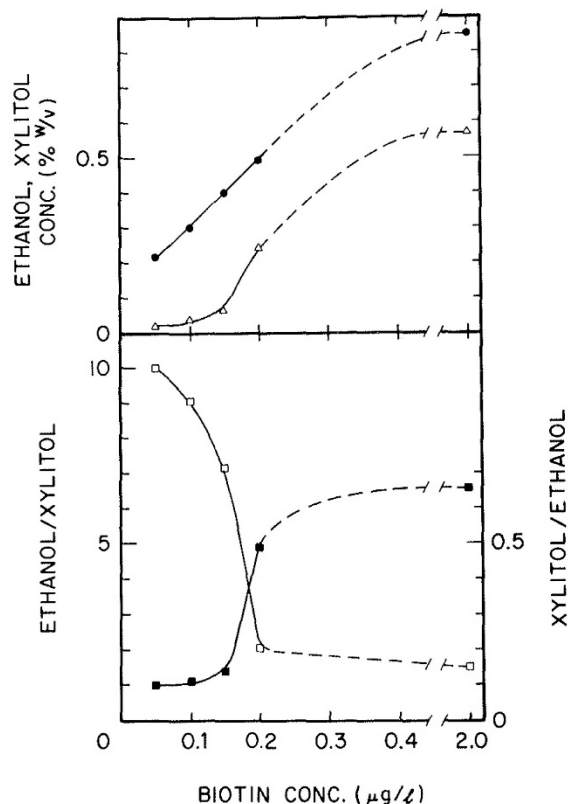


Figure 3. Ethanol and xylitol concentrations in the medium at 100 h in *P. tannophilus* cultures as a function of the initial concentration of biotin. Upper portion: ●, ethanol; △, xylitol. Lower portion: □, concentration ratio of ethanol to xylitol; ■, xylitol/ethanol ratio.

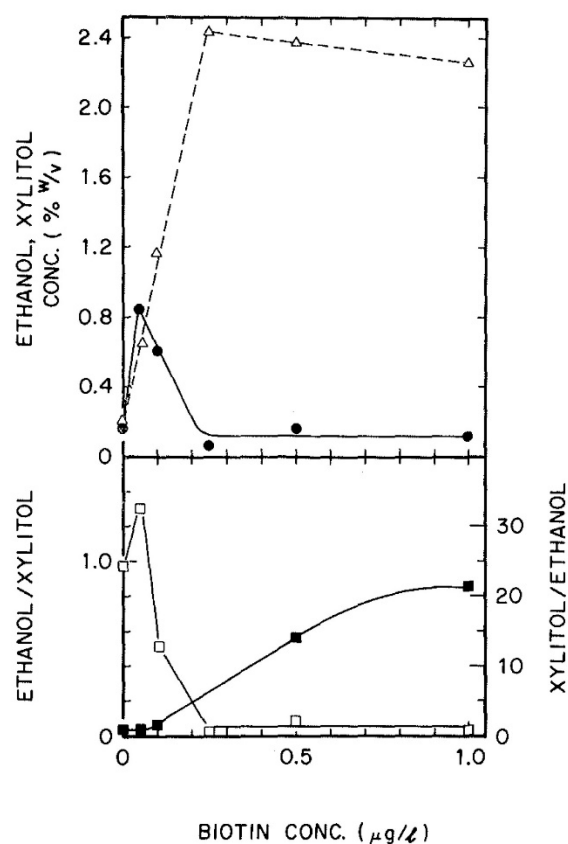


Figure 4. Ethanol and xylitol concentrations in the medium at 100 h in *C. guilliermondii* cultures as a function of the initial concentration of biotin. Symbols as in figure 3.

An aspect of experiments with *C. guilliermondii*, but not *P. tannophilus*, was that the trends found as biotin concentration decreased were readily reproducible, while absolute values varied. For example, in an experiment with *C. guilliermondii*, ethanol to xylitol concentration ratios found at 100 h were, 0.06, 0.16, 0.25, 0.34, and 0.5 for initial biotin concentrations of 0.2, 0.1, 0.075, 0.05, and 0.025 $\mu\text{g l}^{-1}$, respectively. These ethanol to xylitol ratios are lower than those for the results of a similar experiment shown in figure 4, but nevertheless show the same trend: an increased preference to form ethanol at the expense of xylitol as initial biotin concentration decreases. The difference between the two yeasts may reflect higher sensitivity of *C. guilliermondii* to small differences in treatment in different experiments that result in variations in the internal pool of biotin.

A trend common with both organisms over all of the concentrations of biotin studied was that ethanol and xylitol accumulation was associated with the latter stages of the cultures, since at least 80% of product accumulation occurred in conjunction with the last doubling in absorbance.

Discussion

The large differences in product ratio resulting from variations in the initial concentration of biotin are of interest in efforts to modify or control such ratios. It is noteworthy in this connection that there were intervals in some of the *P. tannophilus* cultures when ethanol was present, but not xylitol; figure 1, 50 h, and biotin concentrations of $0.2 \mu\text{g l}^{-1}$ and lower.

Limitation of oxygen can also influence product ratios, but the effects differ from those with biotin. As oxygen limitation increases, xylitol eventually becomes the major product.^{5,6,8} In contrast, ethanol became the preferred product as biotin limitation increased. The ability of limitation by a nutrient other than oxygen to influence product ratio raises the possibility that other nutrients may have potential in this respect. Relatively little is known about the effects of nutrients on the production of ethanol from D-xylose. Most studies employ complex media and some strains produce more ethanol in a rich than a defined medium.¹⁵

For all of biotin concentrations used, xylitol and ethanol acted as if they were secondary metabolites in that their accumulation was associated with the latter stages of growth of the cultures. Thus, changing nutrient conditions altered the relative amounts of two ostensibly secondary metabolites. Changes in the nature of secondary metabolites in response to medium composition has been reported.¹⁷

The changes in ratio of ethanol and xylitol concentration as initial biotin concentration decreased were generally in the same direction for the two organisms studied even though in high biotin they had differing tendencies to produce ethanol and xylitol. The similar effect of biotin limitation in these organisms suggested that the mechanism of biotin limitation on product ratio was the same in both cases. Biotin serves as a prosthetic group for carboxylases, and it is unclear why its limitation results in relatively more ethanol accumulation.

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